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Effect of Human Urinary Thrombomodulin on Endotoxin-Induced Intravascular Coagulation and Pulmonary Vascular Injury in Rats

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Adult respiratory distress syndrome (ARDS) and disseminated intravascular coagulation (DIC) are serious complications of sepsis. Thrombomodulin, an important endothelial anticoagulant, binds thrombin to generate activated protein C (APC). To determine whether thrombomodulin purified from human urine (urinary thrombomodulin, UTM) is useful for the treatment of DIC and ARDS in sepsis, we examined the effect of UTM on endotoxin (ET)-induced coagulation abnormalities and pulmonary vascular injury in rats. Intravenous administration of UTM prevented the ET-induced pulmonary accumulation of leukocytes and the increase in pulmonary vascular permeability, as well as ET-induced histological changes such as leukocyte infiltration and pulmonary interstitial edema. On the other hand, dansyl-Glu-Gly-Arg-chloromethyl ketone-treated factor Xa (DEGR-Xa), a selective inhibitor of thrombin generation, did not prevent these effects of ET. UTM did not prevent ET-induced pulmonary accumulation of leukocytes and pulmonary vascular injury in rats pretreated with DEGR-Xa. Our findings suggest that UTM attenuates ET-induced coagulation abnormalities and pulmonary vascular injury. Furthermore, the latter effect may be dependent on the capacity of UTM to activate protein C. Am. J. Hematol. 54:118–123

Key words: urinary thrombomodulin; activated protein C; disseminated intravascular coagulation; sepsis; adult respiratory distress syndrome; activated leukocytes

INTRODUCTION

Adult respiratory distress syndrome (ARDS) is a particularly important septicemia-related complication because it is associated with a high mortality [1,2]. Because thrombin increases vascular permeability [3], and because intravascular fibrin formation may contribute to the accumulation of leukocytes in the lung [4], disseminated intravascular coagulation (DIC) also may be a precursor of, or a precipitating factor for, ARDS [5,6]. Both DIC and ARDS are thought to be related to the action of cytokines or other inflammatory mediators derived from activated leukocytes [7]. Thus, anticoagulant therapy in patients with DIC may not only ameliorate thrombin generation, but also may prevent the development of the pulmonary vascular injury that can result in ARDS.

Thrombomodulin is an endothelial cell-surface membrane glycoprotein that forms a high-affinity noncovalent complex with thrombin and acts as a cofactor for thrombin in the activation of protein C, enhancing the rate of formation of activated protein C (APC) by >1,000-fold [8]. APC exerts an anticoagulant effect by inactivating factor Va and factor VIIIa [9,10], and a profibrinolytic effect by inactivating plasminogen activator inhibitor-1 [11]. Cytokines such as tumor necrosis factor-α (TNF-α) and

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interleukin-1β have been found to reduce the level of thrombomodulin in cultured endothelial cells to approximately 50% of that in untreated cells [12]. Thus, it is possible that a cytokine-induced decrease in endothelial thrombomodulin may contribute to the development of DIC in patients with septicemia.

Small amounts of thrombomodulin are present in human plasma and urine in their soluble forms [13]. Some of these molecules act as thrombin cofactors in the activation of protein C [14]. Recently, urinary thrombomodulin (UTM) was purified, and has been shown to prevent coagulation abnormalities induced by tissue factor in rats [15]; however, its usefulness in the prevention of endotoxin (ET)-induced coagulopathies and pulmonary vascular injury has not been proven.

In the present study, we examined the effects of UTM on ET-induced coagulation abnormalities and pulmonary vascular injury in rats in order to determine whether UTM may be useful for treating patients with sepsis.

MATERIALS AND METHODS

Materials

UTM was a generous gift of Mochida Pharmaceutical Co. (Tokyo, Japan). UTM was purified from healthy male urine as previously described [15]. Bovine serum albumin was purchased from Sigma (St. Louis, MO); endotoxin (ET) (lipopolysaccharide, *Escherichia coli*, serotype 055:B5), from Difco (Detroit, MI); dansyl-glutamylglycylarginyl-chloromethyl ketone, from Calbiochem (San Diego, CA); and ^{125}I-Bolton-Hunter reagent, from Amersham International (Buckinghamshire, UK). All reagents used were of analytical grade.

Preparation of Dansyl-glutamylglycylarginyl-chloromethyl Ketone-Treated Factor Xa

Factor X was purified from human plasma [16] and activated with Russell's viper venom [17], as previously described. Activated factor X was inactivated by incubation with a 20-fold molar excess of dansyl-glutamylglycylarginyl-chloromethyl ketone for 30 min at 25°C, after which the mixture was subjected to extensive dialysis against a solution containing 20 mM Tris-HCl, pH 7.4, and 100 mM NaCl. Dansyl-glutamylglycylarginyl-chloromethyl ketone-treated factor Xa (DEGR-Xa) has been shown to selectively inhibit thrombin generation by competing with intact factor Xa in prothrombinase complex formation [18]. DEGR-Xa showed no clotting activity and prolonged activated partial thromboplastin time in a concentration-dependent manner (data not shown).

Animal Model of ET-Induced Acute Pulmonary Vascular Injury

The study protocol was approved by the Kumamoto University Animal Care and Use Committee, and the care and handling of animals were in accordance with the National Institutes of Health guidelines. Adult pathogen-free male Wistar rats (body weight, 180–220 g) (Nihon SLC, Hamamatsu, Japan) received intravenous infusions of ^{125}I-labeled bovine serum albumin (specific activity, 3.0×10^{14} cpm/mol) prepared with Bolton-Hunter reagent (2.0×10^5 cpm/kg), 5 min before an intravenous bolus dose (5 mg/kg) of ET was injected into the tail vein. UTM or DEGR-Xa was administered intravenously 30 min prior to injection of ET. At specified times after administration of ET, animals were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg) and exsanguinated via the abdominal aorta. Blood samples were collected in tubes containing a 0.1 volume of 3.8% sodium citrate. Blood was centrifuged at 2,000*g* for 10 min. Control animals received saline only. The lung vasculature was perfused through the right cardiac ventricle with 10 ml of saline. The lungs then were removed and weighed, and the amount of radioactivity remaining within the tissue was measured with a gamma scintillation counter (model 5130, Packard Instruments, Downers Grove, IL). ET-induced pulmonary vascular damage was assessed using the permeability index (the ratio of the amount of radioactivity present in 1 g of lung tissue to the amount of radioactivity present in 1 ml of blood obtained at time of death) [19].

Lung Myeloperoxidase Activity

Pulmonary accumulation of leukocytes was evaluated by measuring lung myeloperoxidase (MPO) activity, as previously described [20]. In brief, lung samples were homogenized using 6 ml of homogenizing buffer containing 0.1 M phosphate buffer, pH 6.0, and 1% hexadecyltrimethylammonium bromide. The homogenate then was sonicated and centrifuged at 4,500*g* for 30 min at 4°C. The supernatant was tested for myeloperoxidase activity. The test sample (0.1 ml) was mixed with 0.6 ml of 0.05 M phosphate buffer containing 0.104 mg/ml o-dianisidine dihydrochloride and 0.0042% hydrogen peroxide, pH 6.0. The change in absorbance at 460 nm was measured over 1 min at 25°C in a spectrophotometer (DU-54, Beckman, Irvine, CA). One unit of enzyme activity was defined as the amount of MPO that causes a change in absorbance of 1.0/min at 460 nm, and was expressed as units per g of lung weight.

Histopathologic Studies of Lungs

Histopathologic examination of the lungs was performed 6 hr after administration of ET (5 mg/kg). Saline, UTM (1 mg/kg), or DEGR-Xa (10 mg/kg) were administered intravenously 30-min before administration of ET. Samples were fixed with 10% formalin and embedded in paraffin, sectioned into 6-μm pieces, and stained with hematoxylin-eosin. Samples were analyzed by a pathologist in a blinded fashion.

Assays

The plasma concentration of fibrinogen was determined as the amount of clottable protein, according to the method of Clauss [21]. Fibrin and fibrinogen degradation products (E) (FDP (E)) were measured in serum samples of rats with the latex agglutination assay, as previously described [22].

Statistical Analysis

Data are presented as the mean ± SD. Data were analyzed by analysis of variance and Scheffe's post hoc test. $P < 0.05$ was accepted as statistically significant.

RESULTS

Effects of UTM and DEGR-Xa on ET-Induced Pulmonary Accumulation of Leukocytes, Pulmonary Vascular Injury, and Coagulation Abnormalities

Pulmonary accumulation of leukocytes, as evaluated by determining lung MPO activity, and pulmonary vascular permeability to ^{125}I-labeled bovine serum albumin increased with time after administration of ET, and peaked at 90 min and at 6 hr of ET administration, respectively [23]. Intravenous administration of UTM (1 mg/kg) significantly blocked the ET-induced increase in pulmonary leukocyte accumulation and subsequent pulmonary vascular injury, whereas intravenous administration of DEGR-Xa had no effect (Fig. 1A,B).

Intravenous administration of UTM prevented both the ET-induced decrease in plasma fibrinogen and the increase in serum FDP (E) observed 6 hr after ET administration in a dose-dependent manner (Fig. 1C,D). Similarly, intravenous administration of DEGR-Xa also prevented these ET-induced coagulation abnormalities in a dose-dependent fashion (Fig. 1C,D). Intravenous administration of DEGR-Xa (10 mg/kg) and UTM (1 mg/kg) prevented neither the ET-induced pulmonary accumulation of leukocytes nor the increase in pulmonary vascular permeability (Fig. 1A,B). The decrease in fibrinogen concentration and the increase in serum concentration of FDP (E) induced by ET at 6 hr were significantly inhibited in animals receiving UTM and pretreated with DEGR-Xa (Fig. 1C,D).

These findings suggest that UTM inhibits ET-induced pulmonary vascular injury by activating protein C, and not by inhibiting thrombin.

Effects of UTM and DEGR-Xa on Changes in Pulmonary Histology Induced by ET

Microscopic observation of lung tissue 6 hr after administration of ET revealed edematous changes and infiltration of granulocytes in the interstitial spaces (Fig. 2B). These changes were not present in lung tissue from ani-

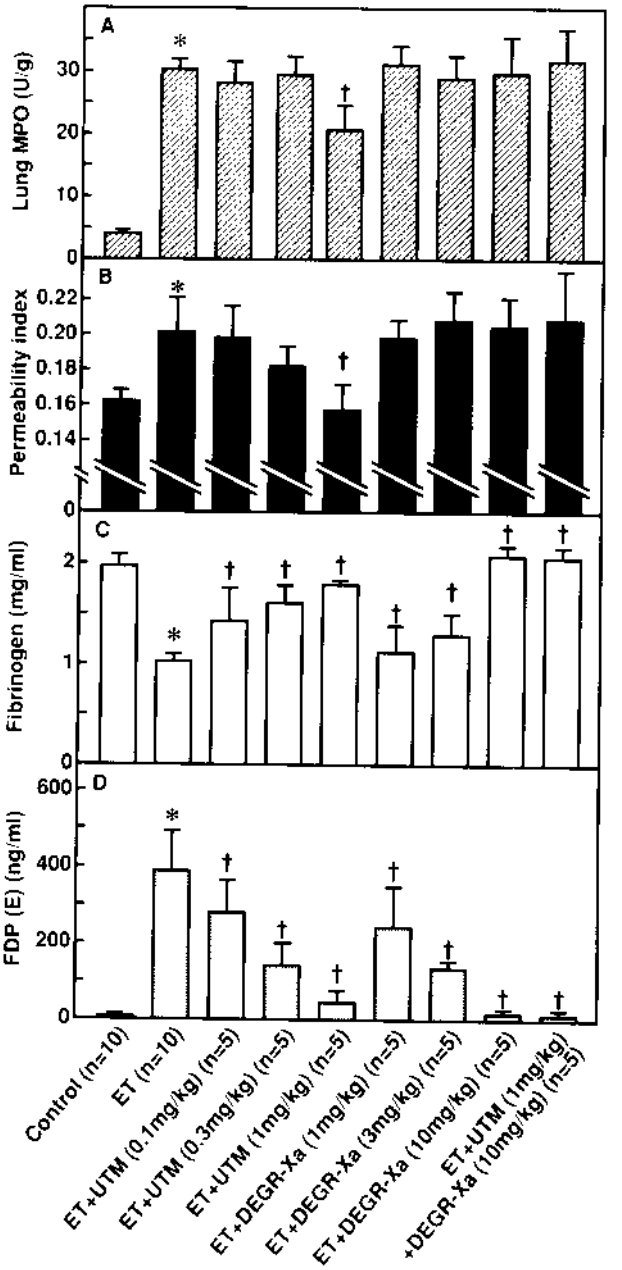

Fig. 1. Effects of UTM, DEGR-Xa, and UTM with DEGR-Xa on pulmonary vascular injury, pulmonary accumulation of leukocytes, plasma concentration of fibrinogen, and serum concentration of FDP (E) in ET-treated rats. UTM or DEGR-Xa were administered intravenously 30 min prior to intravenous injection of ET (5 mg/kg). A: Pulmonary myeloperoxidase activity was determined 90 min after ET administration. Pulmonary vascular permeability index (B), plasma levels of fibrinogen (C), and serum levels of FDP (E) (D) were determined 6 hr after administration of ET. Control animals received 0.9% NaCl instead of ET. Data are expressed as mean ± SD. Numbers in parentheses indicate number of animals in each experiment. *$P < 0.01$ vs. control; †$P < 0.01$ vs. ET.

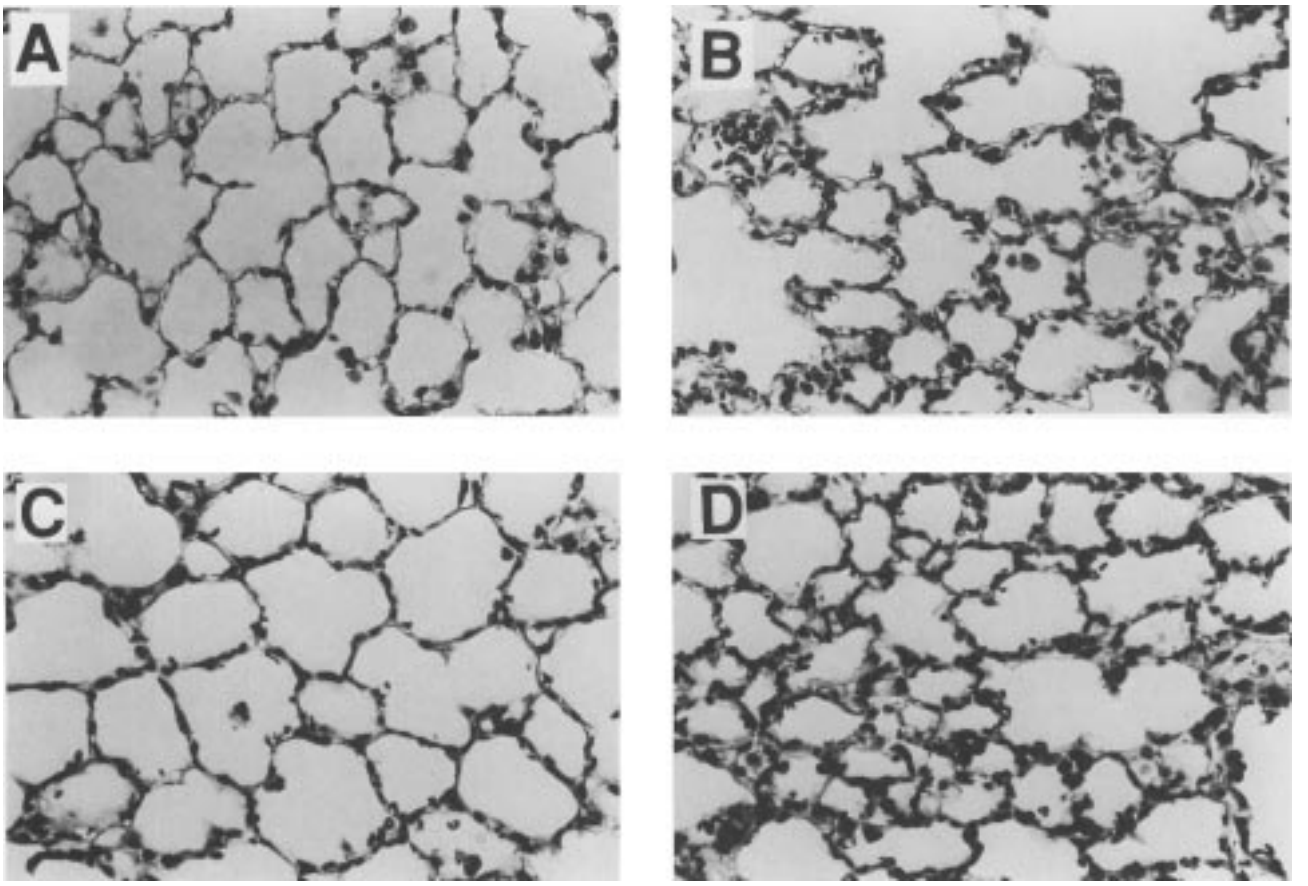

Fig. 2. Microscopic observation of lung tissue from animals treated with saline (A, ×400), ET (B, ×400), ET plus UTM (C, ×400), and ET plus DEGR-Xa (D; ×400). Histopathologic examination of lungs was performed 6 hr after ET (5 mg/kg) administration. Saline, UTM (1 mg/kg), or DEGR-Xa (10 mg/kg) were administered intravenously 30 min before injection of ET. Interstitial edema formation and infiltration of granulocytes were observed in ET-treated animals (B), while these changes were not observed in saline-treated animals (A). UTM markedly reduced interstitial edema and granulocytic infiltration (C), whereas DEGR-Xa did not reduce interstitial edema or granulocytic infiltration (D).

mals treated with saline alone (Fig. 2A). Intravenous administration of UTM (1 mg/kg) markedly reduced interstitial edema and granulocytic infiltration (Fig. 2C), whereas intravenous administration of DEGR-Xa (10 mg/kg) did not reduce these ET-treated changes (Fig. 2D). ET-induced histological changes were not reduced by UTM (1 mg/kg) in animals pretreated with DEGR-Xa (10 mg/kg) (data not shown).

DISCUSSION

In the present study, UTM was demonstrated to prevent ET-induced coagulation abnormalities and pulmonary vascular injury. UTM has also been shown to prevent tissue factor-induced DIC in rats [15]. Kumada et al. [24] demonstrated that thrombomodulin purified from murine lung prevented thrombin-induced thromboembolism in mice. Recombinant human soluble thrombomodulin also prevents tissue factor- or ET-induced DIC in rats [25,26]. These observations are consistent with our current observations. These preventive effects may be dependent on direct inhibition of thrombomodulin on thrombin, or on its ability to generate activated protein C, which inactivates factors Va and VIIIa [9,10].

In addition to prevention of ET-induced coagulation abnormalities, pulmonary accumulation of leukocytes and subsequent pulmonary vascular injury also were prevented by UTM in this study. Although DEGR-Xa almost completely blocked ET-induced coagulation abnormalities, it did not affect the ET-induced pulmonary accumulation of leukocytes or subsequent pulmonary vascular injury, suggesting that these coagulation abnormalities alone are not responsible for ET-induced pulmonary vascular injury in this animal model. This observation is consistent with our previous report demonstrating that ET-induced pulmonary vascular injury is not mediated by these coagulation abnormalities but by activated leukocytes [27]. Thus, UTM may prevent ET-induced pulmo-

nary vascular injury by inhibiting activated leukocytes. Since the preventive effects of UTM on pulmonary accumulation of leukocytes and subsequent vascular injury were not observed in animals pretreated with DEGR-Xa, and since APC has been shown to prevent ET-induced pulmonary vascular injury by inhibiting leukocytes [23], it is likely that UTM prevents ET-induced pulmonary vascular injury primarily by generating APC. Consistent with this notion are our previous reports demonstrating that recombinant TM prevented ET-induced pulmonary vascular injury by inhibiting activated leukocytes via protein C activation in rats [19,23]. Taylor et al. [28,29] also found that APC inhibited activation of leukocytes. In their study, DEGR-Xa prevented *Escherichia coli*-induced coagulopathy, but did not prevent organ damage or shock, whereas APC prevented both the coagulopathic and lethal effects of ET. Snow et al. [30] demonstrated that APC exerts a cytoprotective effect against tissue damage induced by reperfusion-ischemia, a setting in which activated leukocytes are thought to play a role. Esmon et al. [31] also reviewed the antiinflammatory effects of APC. These findings together with the present results suggest that the in vivo protective effect of UTM against ET-induced pulmonary vascular injury may be related to the inhibitory effect of APC on activation of leukocytes.

Although the precise mechanism for the antiinflammatory effects of APC has not been fully elucidated, the suppressive effect of APC on ET-induced production of cytokines in vitro and in vivo has been demonstrated [32]. It is possible that the suppressive effect of APC is related to its inhibition of endotoxin/CD14 coupling, by which ET may stimulate the production of cytokines [33]. Grinnell et al. [34] demonstrated that APC inhibits E-selectin-mediated cell adhesion. This effect is not mediated by the serine protease activity of APC; instead, the carbohydrate moieties of APC competitively inhibit the interaction between E-selectin and the sialylated Lewis X antigen of leukocytes. However, we previously demonstrated that active site-blocked APC prevented neither ET-induced pulmonary accumulation of leukocytes nor subsequent pulmonary vascular injury [23], suggesting that the inhibitory effect of APC on leukocyte activation may be mediated by its serine protease activity.

These findings together with the present results suggest that UTM may be useful in treating patients with sepsis-associated DIC and pulmonary vascular injury.

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